17. Vitamin Methods

3. The Reliability of the Method for Estimating Vitamin C by Titration against 2:6-Dichlorophenolindophenol.
1. Control Tests with Plant Tissues

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Until about 8 years ago the only method for determining vitamin C was by means of biological tests on guinea-pigs. In consequence, knowledge about the distribution and behaviour of the vitamin accumulated slowly and with difficulty, and it was not until a chemical method had been introduced that progress became more rapid.

The first indication that certain fruit juices and animal fluids were able to decolorize a redox dye of the indophenol class, viz. the 2:6-dibromo derivative, was given by Mansfield Clark and his colleagues [Cohen *et al.* 1924]. Zilva [1927; 1932] later noted that certain concentrates of vitamin C reduced the parent substance, phenolindophenol, but he attributed the reaction not to vitamin C itself but to some associated 'protective substance'. Tillmans and his coworkers [e.g. Tillmans, 1930; Tillmans *et al.* 1932, 1, 2] made the important observation that the ability of foodstuffs to reduce 2:6-dichlorophenolindophenol frequently went roughly parallel with their known antiscorbutic potencies; there were, however, important exceptions. By working out conditions for the preliminary extraction of the foodstuff, and by carrying out the titration itself rapidly and in acid solution, Harris & Ray [1933, 1] and Birch *et al.* [1933] converted the principle of the Tillmans reaction into a specific and accurate test for the quantitative estimation of ascorbic acid in foodstuffs. The preliminary extraction process ensured the complete removal of the vitamin C from the tissue without incurring loss by oxidation and also separated various interfering materials; the rapid titration in acid reaction prevented the inclusion in the titre of non-specific reducing substances, notably glutathione, which would otherwise be the cause of serious error.

This method has been used for a wide variety of purposes, and it now seems advisable to review certain aspects of the procedure and to give further evidence which we have secured relating to its accuracy and specificity. We propose in the present paper to deal with plant tissues only.

The reliability of this chemical method of estimation has already been confirmed for many fruits and vegetables (as well as for animal tissues), as a result of numerous direct comparisons against biological assays [e.g. Harris & Ray, 1933, 1, 2, 4; Lund *et al.* 1934; Ray, 1934; Levy & Fox, 1935; Olliver, 1936; Mathiesen & Aschehoug, 1937; Mathiesen, 1938; 1939; Watson & Kon, 1936; Maclinn & Fellers, 1938; Jacobsen & Faulenborg, 1939]. In addition, numerous chemical tests have been carried out without direct biological comparison, and here again the results are generally in excellent agreement with the reputed biological values. In Table 1 the average results of some hundreds of tests which we have carried out on freshly gathered fruits and vegetables are set out to show their good accord with the accepted biological activities.

A small minority of workers have made observations which might at first sight appear to set limits to the accuracy of the chemical test. It is to clear up such doubts that this paper is written. The supposed objections to the method are threefold: (1) it fails to detect the hypothetical 'combined ascorbic acid' presumed to be present in various plant tissues; (2) certain non-specific but unspecified interfering substances, distinct from ascorbic acid, may also reduce the indicator; (3) significant amounts of the reversibly oxidized but still biologically active vitamin (dehydroascorbic acid) may fail to be estimated. Our own

Table 1. *Ascorbic acid in fruits and vegetables; agreement*
between chemical and biological values

Name	Chemical value, mg./100 g. (arranged in descending order) Average	Range	Biological values, as recorded before, 1933 'Sherman units'* (from tabulations of Daniel & Munsell [1937])	Name	Chemical value, mg./100 g. (arranged in descending order) Average	Range	Biological values, as recorded before 1933 'Sherman units'* (from tabulations of Daniel & Munsell [1937])
Hips	520	222–790	—	Orange (Seville)† juice	34	19–45	—
Black currant	231	79–344	200	Pea	30	12–49	33
Kale	145	80–193	—	Potato, new	30	26–39	33
Horseradish	136	127–144	200	Broad bean	27	22–29	—
Brussels sprouts	112	65–146	50–100	Turnip	26	17–36	14–33
Turnip top	100	93–114	—	Raspberry	25	21–37	20
Cabbage	90	50–182	57–150	Tomato	24	12–42	20–67
Haws	92	64–125	—	Lettuce	20	5–30	4–100
Cauliflower (white head)	70	49–92	—	Runner bean	20	17–22	—
Broccoli (white head)	69	57–91	—	Blackberry	14	3–24	—
Asparagus	67	32–115	10–50	Bilberry	13	11–15	—
Spinach	65	30–118	14–100	Samphire	13	13–14	—
Watercress†	61	58–72	100	Apples (various)	12	4–17	2–33
Strawberry	61	44–93	26–100	Potato, old†	11	6–17	7–20
Orange (sweet)† juice	58	33–77	51–65	Stringless bean	11	7–14	—
Lemon† juice	46	29–60	53–67	Banana†	10	6–15	10–17
Red currant	46	40–51	33	Rhubarb	8	7–9	—
White currant	44	38–52	—	Parsnip†	7	5–9	—
Grapefruit† juice	40	28–64	50–100	Onion	7	5–10	<20
Gooseberry	40	22–55	50	Carrot	6	1–13	3–10
Swede	40	30–49	—	Greengage	5	3–7	<16
Elderberry	35	28–40	—	Melon†	3	2–3	—
Loganberry	34	20–48	—	Mushroom	3	2–4	0–<10
				Pear	3	2–3	0–10
				Plum	2	<1–3	<5–10

* We have purposely refrained from attempting to convert Sherman units into mg./100 g. The general correspondence between the chemical and biological values in the two descending scales is sufficiently apparent.

† All values relate to mature fruit and vegetables, freshly picked except those marked by a dagger, which had been stored after picking.

experiments with plant tissues convince us that, provided that the correct procedure is adhered to for the titration, these difficulties either do not exist (i.e. as far as concerns the 'combined ascorbic acid' or the 'interfering substances') or else are normally of little or no practical importance and can in any case be accurately allowed for (i.e. dehydroascorbic acid). We have been struck by the fact that those few workers who have experienced these difficulties have failed to follow the procedure recommended. Thus it is important that during the preliminary extraction of the vitamin it should be protected against destruction by oxidases. This was accomplished in the original method of Harris & Ray by working with a small specimen, carrying out a rapid extraction with trichloroacetic acid and titrating the extract without delay. (In the more recent modification the same object is achieved by the addition of a proportion of metaphosphoric acid which stabilizes the vitamin even more effectively.) This danger of oxidation does not appear to have been always recognized, as for example when large specimens have been taken and submitted to long and laborious processes of extraction [see McHenry & Graham, 1935, 1]. This, we believe, accounts for the unduly low values which these workers record for the raw foods (the so-called 'free ascorbic acid'), as well as for the apparent increase after boiling (which, in our opinion, can be largely attributed to the concurrent inactivation of the enzymes). Similarly, the large amounts of the dehydroascorbic acid sometimes reported in fresh plant tissues we consider to be generally an artefact produced by oxidation.

It seems advisable for us to set out in detail the precise working directions which we follow in carrying out the test. This forms the first part of the paper. Later sections deal in turn with the following topics: (1) the results of new assays illustrating the agreement between the biological determinations and chemical titrations; (2) an examination for supposed interfering substances (e.g. heated sugar derivatives); (3) the significance of reversibly oxidized vitamin (dehydroascorbic acid); (4) the question of 'combined ascorbic acid'.

A. SPECIAL ASPECTS OF THE PROCEDURE IN THE CHEMICAL METHOD

It must be emphasized at the start that it is essential that four major precautions should be observed if accurate results are to be obtained in the titration method. These are: (1) representative sampling; (2) complete extraction; (3) prevention of oxidation; (4) rapid performance of the titration itself.

(1) *Sampling.* It is imperative that sampling should be representative of the whole quantity of material under examination. This may be difficult because of the surprisingly wide variations in the content of ascorbic acid in different individual fruits and vegetables in a batch, as well as in different parts of the same individual (see, for example, Table 2, and cf. Olliver [1938]). We have found it preferable to carry out estimations on several different samples from one batch and to take the average value, rather than to rely upon single tests on a large and supposedly representative sample. Any fine chopping, shredding or mincing of the material, with the supposed object of obtaining a homogeneous specimen, must be condemned, because intracellular oxidases will thus be set free from many vegetable tissues, with a consequent rapid disappearance of the ascorbic acid. Examples showing the seriousness of this source of error are given in Table 4. If some cutting is necessary, e.g. as with cabbage and other leafy vegetables, the aim should be to reduce it to a minimum by taking several relatively large pieces and combining them, and then repeating the estimation on another similar selection of pieces.

(2) *Complete extraction.* Complete extraction is difficult if too large an amount of the material is taken or if the tissue is not sufficiently well ground. For this reason we believe it to be a mistake to attempt to carry out the titration on a macro scale, as has

Table 2. *Variations in ascorbic acid content of black currants, depending on size of fruit. All specimens picked on one day from similar bushes*

	Ascorbic acid, mg./100 g.		
Colour of berry	Small berries (0·14–0·27 g.)	Medium sized berries (0·30–0·47 g.)	Large berries (0·60–0·94 g.)
Red	271	262	—
Purple	315	256	255
Black	296	242	219

Table 3. *Destruction of ascorbic acid by oxidases set free on grinding. Protective action of acid*

Time after grinding before addition of acid min.	Ascorbic acid, mg./100 g.		Time after grinding before addition of acid min.	Ascorbic acid, mg./100 g.		Time after grinding before addition of acid min.	Ascorbic acid, mg./100 g.	
	Normal method	After reduction by H_2S		Normal method	After reduction by H_2S		Normal method	After reduction by H_2S
Broccoli head (freshly harvested)			Potatoes (King Edwards, stored 1 month)			Cortex of Bramley apple		
0	61	61	0	13	13	0	11	11
½	36	37	⅛	10	12	½	9	11
1	23	24	¼	10	12	¾	8	9
1½	9	21	½	8	11	1	7	9
2	5	16	¾	5	9	1½	4	7

Table 4. *Effect of shredding or chopping on vitamin C in plant tissues*

Description and treatment immediately before extraction	Ascorbic acid found on titration mg./100 g.	Description and treatment immediately before extraction	Ascorbic acid found on titration mg./100 g.
Brussels sprout leaf:		Carrots, young:	
Not cut	71	Not cut	20
Coarsely cut (strips 1 in. wide)	71	Shredded, left 5 min.	12
Finely shredded	68	Shredded, left standing 1 hr.	9
Cauliflower leaf:		Carrots, old:	
Not cut	162	Not cut	8
Coarsely cut (strips 1 in. wide)	162	Shredded	7
Finely shredded	138	Shredded, left standing 1½ hr.	5

sometimes been done, in place of the original micro-technique recommended by Birch *et al.* [1933]. Another point to which attention has to be devoted is the need for special care with woody tissues, which are difficult to extract completely and may often be low in ascorbic acid. Examples of erroneous values due to incomplete extraction are cited in the following pages.

(3) *Prevention of oxidation.* As has been mentioned above, the necessity for retarding the oxidation brought about by the natural oxidases of the tissues (or even by the simple access of air) has not been realized by all workers, some of whom have adopted complicated processes of extraction entailing the use of unduly large amounts of specimen and ex- tractant. Apart from the advisability of rapid extraction (which implies the use of a small sample and thorough grinding) four other points should be stressed under this heading: (*a*) The sampling should be done in such a way as not to liberate the enzyme (see above). (*b*) Copper-free distilled water should be used for all determinations.* (*c*) In order to delay oxidation the acid must be added to the sample and its surface covered before the grinding is begun [cf. also Thornton, 1938]. The rapidity of the oxidation if grinding is done in absence of the protective acid is shown by results in Table 3. Risk of destruction by oxidation may also occur if too large quantities of material are taken, since immediate intimate contact between acid and tissue is thereby prevented. (*d*) The presence of metaphosphoric acid [Fujita & Iwatake, 1935; Musulin & King, 1936; Harris, 1937] materially assists in retarding the oxidation catalysed by these enzymes or by Cu, and it should invariably be used in the extraction.

(4) *Rapidity of titration.* The reaction between ascorbic acid and the dyestuff is virtually instantaneous. The titration should be completed and the end-point reached within about 1 min., otherwise non-specific substances may possibly be included which decolorize the dye more slowly. An immediate end-point is therefore to be aimed at, and any gradual fading of the dye has to be avoided.

(5) *Acid reaction.* Some workers, following Tillmans's original procedure, have attempted to estimate ascorbic acid by titration in neutral solution, instead of at a more strongly acid reaction. Results may be almost identical for some citrous fruits, because interfering substances chance not to be present in appreciable amounts; but, as a general method, titration at neutral reaction is liable to lead to gross errors, especially in the case of animal tissues, in which glutathione would be the principal source of trouble.

(6) *'Reversed' titration.* The titrimetric method of Bessey & King [1933] is virtually identical with that described somewhat earlier by Harris & Ray and Birch, Harris & Ray, i.e. extraction is in trichloroacetic or acetic acid, oxidation is avoided and the titration which follows is performed rapidly and in an acid medium. The only obvious difference is that the Cambridge workers had recommended a 'reversed' titration, the unknown being titrated against a fixed amount of dye instead of *vice versa.* With clear colourless extracts

* The modified procedure, employing metaphosphoric acid, largely guards against this danger of inactiva- tion by Cu; nevertheless, the precaution of using Cu-free reagents still remains a useful one.

probably either alternative is almost equally satisfactory, but with moderately pigmented extracts it is the experience of these and of other laboratories that the end-point is easier with our method of 'reversed' titration: the first discharge of the faint pink colour is more readily detected than its first appearance. The difference of procedure is perhaps of little importance. With either method it is an advantage to have two control tubes, one containing the dye just before and the other just after discharge, both in the presence of a volume of extract equal to that being titrated.

The working directions, embracing all the safeguards just discussed, and also, for the sake of ease in manipulation, incorporating several minor modifications, are set out in detail below.

B. WORKING DIRECTIONS

Reagents. Blank tests should be done on all the reagents, particularly the trichloro-acetic acid, to ensure that they are free from impurities which may reduce the dye. A good-quality brand of trichloroacetic acid should be used, and if necessary repurified till it is without action on the dye. Distilled water should be recently boiled, and this and all other reagents should be Cu-free (cf. footnote, p. 158). Solutions of metaphosphoric acid have to be made up daily, as conversion into orthophosphoric acid occurs on standing.

Preparation and standardization of dye solution. Dissolve 20 mg. of repurified 2:6-dichlorophenolindophenol in 25 ml. of cold, recently boiled Cu-free distilled water and filter. This dye solution must be restandardized daily against a freshly prepared solution of pure ascorbic acid (the activity of which should be cross-checked occasionally by titration against 0·01 N iodine). To prepare the standard solution of ascorbic acid, weigh out accurately* 10·00 mg. of a repurified specimen, wash it into a 250 ml. measuring flask with 25 ml. 20 % metaphosphoric acid, and make up to the mark with redistilled water. Approximately 0·5 ml. of this solution ($\equiv$0·02 mg. ascorbic acid) will be needed in the titration, which is carried out against 0·05 ml. of the dye, and performed in the same manner as described below for foodstuffs with of course the omission of the preliminary grinding and extraction. The dye solution slowly deteriorates and should not be kept for longer than 10 days, otherwise an indefinite end-point may result.

Preparation of 'strong acid mixture'. To 5 parts 20 % trichloroacetic acid add 2 parts 20 % metaphosphoric acid (freshly prepared, see above). In our more recent work we have substituted metaphosphoric acid, 20 %, for the 'strong' mixture of metaphosphoric and trichloroacetic acid, since the latter acid is at present less readily obtainable. There appears to be no loss in accuracy—certainly with vegetable tissues with which we have been principally concerned—and the risk of oxidation is probably further diminished.

Choice of sample. A representative specimen is first selected (see above, regarding choice of material and avoidance of chopping, shredding and mincing) and accurately weighed. If it has been impossible to avoid a certain amount of cutting of the tissue it is essential that the weighing be done rapidly; if necessary the material may be weighed under the acid to prevent oxidation. The amount taken can vary somewhat according to the activity. A suitable quantity, when working with material of average activity, say fresh peas, is about 5–10 g. ($\equiv$1·5–3 mg. ascorbic acid). It is recommended that not less than 2 g. nor more than 20 g. of any material should be used. The aim is to prepare an extract of such concentration that 0·5–1·0 ml. of it is required for titration against 0·05 or 0·10 ml. of dye solution. If the material is very poor in ascorbic acid it is advisable to take less of the dye for the eventual titration rather than to weigh out an unduly large quantity for estimation. Alternatively, if the material is unexpectedly potent, it can be further diluted, if found necessary, at a later stage when the actual

* If a micro-balance is not available weigh out instead 50 mg.; wash into a 200 ml. flask with 20 ml. 20 % metaphosphoric acid; make up to mark with distilled water; take 20 ml. of this solution and dilute to 200 ml. with a further 18 ml. 20 % metaphosphoric acid and distilled water; titrate against 0·05 ml. of dye: approximately 0·8 ml. ($\equiv$0·02 mg. ascorbic acid) should be needed.

titration is carried out; this is preferred to the use of a smaller weighed specimen, which would be less easy to grind up effectively and probably less representative.

Preparation of extract. Two alternative methods are available for the preparation of the extract, the choice between them depending on the nature of the material under examination. In method 1, extraction is begun with a relatively strong (20 %) solution of acid, and the extract is subsequently filtered and then diluted. In method 2, the diluted acid is used from the start, and centrifuging replaces filtering. Method 1 is used for watery fruits or vegetables (e.g. melons, marrows); any undue dilution of the protective acid by the natural water of the specimen is thereby avoided; moreover, grinding is facilitated since the mixture does not become too 'sloppy'. Method 2 is found more convenient and rapid for most purposes, particularly for bulky, fibrous tissues, with which there might be a risk of incomplete extraction if the smaller volume of strong acid were used.

Extraction method 1. When working with materials whose activities are approximately known we have found it convenient to work to the scheme given in Table 5, which indicates the most suitable quantities of the specimen, extractant and standard dye solution to employ. For an extract of final volume 100 ml., measure out 35 ml. of the 'strong acid mixture' (or 25 ml. of the 20 % metaphosphoric acid) into a graduated

Table 5. *Working directions. Approximate quantities and dilutions suitable for use with materials of varying potencies*

Range of activity, ascorbic acid mg./100 g.	Wt. of sample to be taken g.	Final vol. of extract ml.	Vol. of standard dye solution used ml. (0·05 ml. =approx. 0·02 mg. ascorbic acid)
		(a) Direct titration	
1–2	20	50	0·0125
3–5	20	100	0·0125
6–10	20	100	0·0250
11–15	20	100	0·0500
16–25	15	100	0·0500
26–35	10	100	0·0500
36–70	5	100	0·0500
71–100	5	100	0·100
101–200	5	200	0·100

Range of activity, ascorbic acid mg./100 g.	Wt. of sample to be taken g.	Final vol. of extract ml.	Strength of dye solution (at full strength, 0·05 ml. =approx. 0·02 mg. ascorbic acid)	Vol. of extract used for titration ml.
		(b) 'Chloroform' method		
1–5	20	50	$\frac{1}{4}$	4
6–15	20	50	$\frac{1}{4}$	2
16–30	20	100	$\frac{1}{4}$	2
31–40	20	100	$\frac{1}{4}$	2
41–50	15	100	$\frac{1}{2}$	2
51–70	12	100	$\frac{1}{2}$	2
71–90	15	100	Full	2
91–130	12	100	Full	2
131–165	10	100	Full	2
166–200	8	100	Full	2
201–250	5	100	Full	2

cylinder. To the weighed specimen, placed in a mortar, add roughly one-third of the acid and about 2–5 g. of acid-washed, quartz sand. If the specimen needs preliminary chopping or cutting, this must be done under the acid. Grind rapidly but thoroughly, taking care to ensure full disintegration of tissue and complete extraction. Then add about one-half of the remaining acid. If too much acid is added in the first place some difficulty may be experienced in effecting complete disintegration. Next filter rapidly through fine muslin into a 100 ml. graduated flask, aiding the extraction by pressing the residue well with a glass rod. Return the friable mass to the mortar and repeatedly extract with the remainder of the acid. Wash the residue thoroughly with water, uniting all washings in the flask, until the volume reaches 100 ml. (The final concentration of trichloroacetic acid is now approximately 5 % and that of metaphosphoric acid 2 %, or if metaphosphoric acid alone is used, about 5 %; the total amounts of acid and water used may be varied provided that the final concentration of the former is still approximately 5 %.) Filter the extract through a fluted Whatman no. 1 filter paper and titrate without delay.

Extraction method 2. For a material of average activity (e.g. cabbage) about 5–10 g. of the weighed representative specimen are ground in a mortar, in the presence of about 2–10 g. of sand, with 25–40 ml. 5% metaphosphoric acid. This can be added little by little, but care must be taken to cover the tissue with the acid from the start, to inactivate oxidases. The mixture of extract and residue is transferred to the centrifuge, and, after spinning, the clear liquid is decanted off. The residue is returned to the mortar and reground with the addition of a further 15–20 ml. 5% acid, and the supernatant extract separated once more by spinning and decantation. The process is repeated yet a third time, again using 15–20 ml. of acid. The combined extracts are made up with 5% metaphosphoric acid to a final volume of 75–100 ml., before titration. With a very potent raw material the sample may be reduced to about 2 g. We recommend the use of this simplified process whenever access can be had to a suitable centrifuge. Desiccated products require soaking for some time in the acid before grinding, to render them sufficiently soft for extraction.

Titration against dye. Place a portion of the acid filtrate or extract, prepared by either of the two methods described above, in a 2 ml. microburette, graduated in 0·01 ml. Measure accurately, into a pointed centrifuge tube of about 10 ml. capacity, from a graduated micropipette, a suitable volume of the dye solution (generally 0·05 ml., but sometimes either double or else one-half to one-quarter that volume).* Run the extract rapidly into the tube until the red colour, which the dye assumes in the acid solution, is just discharged. The determination of the end-point is facilitated by comparison with control tubes containing the extract before and after the discharge of the dye (p. 159). The titration should be completed in less than 1 min., one or two rough trial determinations being done first to gain an approximate indication of the titre.† The time occupied by the complete test need not exceed 10–15 min. from the taking of the specimen to the end of the final titration.

Best results are obtained when the amount of extract required is around 0·5–1·0 ml. for 0·05 ml. of standard dye solution. If appreciably more than 1·0 ml. of extract is required, titrate again taking a smaller amount of the dye solution, either 0·025 or 0·0125 ml. If less than 0·5 ml. of the extract is required, dilute it before further titration with some more 5% acid solution until a suitable dilution has been reached.

Macro method. Since many analytical laboratories still do not possess apparatus for micro determinations, it should be added that the titration can if desired be done on a macro scale with the use of an ordinary macro burette. With 0·5 ml. of standard dye about 10 ml. of extract should be needed in the titration; 1·0 ml. or 0·25 ml. of dye can be substituted for extra strong or weak extracts respectively. There is, however, no gain in speed, convenience, or accuracy, and we prefer the micro titration.

Procedure for fruit juices. When examining clear fruit juices, e.g. strained lemon juice or orange juice, simply make up to the required dilution (i.e. 0·5–1·0 ml. of liquid ≡ 0·05–0·10 ml. of standard dye) by the direct addition of acid, the final concentration of the latter being approximately 5%. Filter again if necessary, or remove any precipitate by centrifuging. As most fruits (in contrast with vegetables) are devoid of oxidases, glacial acetic acid (5% of volume) can be substituted for the usual metaphosphoric acid, mixture of trichloroacetic and metaphosphoric acid, if the juice is to be titrated without delay.

With citrous fruits it is probably as accurate to titrate at a more nearly neutral reaction, pH 5–6, since reducing substances other than ascorbic happen not to be present in appreciable amounts; the end-point with the blue dye, i.e. in neutral (or nearly neutral) solution, is rather easier to determine than with the pink form into which it is transformed in more strongly acid solution. (Even direct titration with iodine may give approximately correct results for some citrous fruits.) But it deserves to be emphasized that as a general rule (and especially with animal tissues) titration in neutral solution is a highly dangerous proceeding, owing to the risk of interfering substances being present which reduce the dye rapidly at a high but not at a low pH.

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* Even a quarter-volume of dye (0·0125 ml.), used only with materials of low activity, can be measured with sufficient accuracy from an ordinary micropipette, and we prefer this procedure to the alternative of a dye diluted four times. Micropipettes of 0·1, 0·05 or 0·02 ml. capacity, graduated in each case in 100 divisions, should be used, depending on the amount required. For greater precision, an Agla automatic syringe, with micrometer attachment, may be substituted if desired.

† In view of past misunderstandings, it should be made clear that it is not an end-point which persists for 1 minute which is to be reached, but that the whole titration is to be completed within a minute from the moment when the operator starts to run the extract into the dyestuff until the reading of the burette is taken.

Modified method for coloured extracts. When the extract is deeply pigmented, as for example with black currants and certain other berries, visual determination of the end-point may be difficult. The simplest procedure in such instances is to measure the end-point electrometrically [Birch *et al.* 1933]: all that is needed is a mercury-coated platinum wire dipping into the solution and a suitable arrangement for measuring the potential. The accuracy is even greater than with visual matching (for full details of procedure see Harris *et al.* [1942]). Otherwise the working directions are as already described. If, however, a potentiometer and platinum wire are not available the following method may be used, though the end-point is less sharply defined than that determined electrometrically.

'*Chloroform' process.* First gain an approximate indication of the order of activity of the coloured extract by direct titration, using two control tubes, the first containing the extract with the dye added, and the second the extract after discharge of the dye. Next prepare a fresh extract, adjusting the weight of the sample taken so that 2 ml. of the extract require 0·5–1·0 ml. of the dye. To satisfy this condition the dye solution will probably have to be prepared at a greater dilution than in the normal procedure or the extract will have to be made up more concentrated. Use the amounts of sample, acid and dye recommended in the second half of Table 5. Place 1 ml. of chloroform [McHenry & Graham, 1935, 1] in a long, pointed centrifuge tube, and add 2 or 4 ml. (Table 5) of the filtered, freshly prepared extract. Agitate the upper aqueous layer only by passing through it a stream of O_2-free CO_2 at a medium pace. Add the dye solution from the 2 ml. microburette to within 0·02–0·05 ml. of the required volume. Then allow the two layers to mingle gently by dipping the inlet of gas below the junction of the two layers. The end-point is shown by the development of a definite pink tint in the chloroform layer. (If an emulsion forms it can be broken by centrifuging or rubbing the tube between the hands.) It is very important that the dye after each fresh addition should be well mixed into the extract before the chloroform is brought through the aqueous layer—otherwise, should the dye come into direct contact with the ·chloroform, a colour may appear in the latter which is not discharged by the excess of ascorbic acid still present in the aqueous layer. Occasionally a very faint pink coloration is produced before the true end-point. This can be avoided by taking the colour to a well-defined pink in a preliminary titration, so as to ensure that the true end-point has been reached. Also the extract and the chloroform should not be left standing together in the tube before titration, otherwise a low result may be given. A little experience is needed to gain confidence in working this modification for pigmented extracts. As already recommended, however, the alternative electrical method should always be used if the extra equipment (platinum wire and potentiometer) is available.

Determination of dehydroascorbic acid (reversibly oxidized vitamin C). As mentioned below, the amount of vitamin C present in foodstuffs in the form of dehydroascorbic acid, even during ageing and in stale foods, is generally so small as to be of little or no practical significance. It may however occasionally be desired to estimate it separately. The procedure
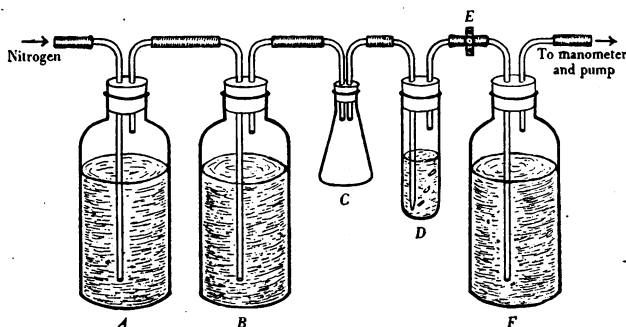

Fig. 1. Apparatus used for removal of H_2S from extract. *A, B* = solution of alkaline pyrogallol; *C* = trap; *D* = extract to be 'de-gassed'; *E* = screw clip; *F* = NaOH.

resembles that already described. First estimate ascorbic acid by the usual method, then estimate ascorbic acid plus dehydroascorbic acid by the following technique. Pass a stream of H_2S gas for 10–20 min. through 25 ml. of the filtered extract prepared as before and contained in a 100 ml. conical flask, stopper the vessel tightly and stand for 12 or 24 hr. (e.g. overnight), preferably in the ice box. Remove the H_2S by bubbling O_2-free N_2

through the solution while it is simultaneously connected with an efficient vacuum pump. (The apparatus employed is shown in Fig. 1; the nitrogen may be obtained from a cylinder, and passed through alkaline pyrogallol before entering the extract, to remove all traces of O_2.) Continue, until a negative reaction for H_2S is given on a portion of the liquid by the nitroprusside-ammonia test or by testing the vapour from it with lead acetate paper (generally within about 20–30 min.). Make up to volume if necessary, to allow for any evaporation. Titrate as before. The difference between the two titrations gives the amount of dehydroascorbic acid.

This procedure for the effective removal of the H_2S by simultaneous gassing with N_2 and connexion with a vacuum was suggested to us by Dr L. W. Mapson. Previously we had been in the habit of using N_2 without any concurrent evacuation. Under these circumstances it may be difficult to remove all traces of H_2S after even 8–10 hr. treatment with a rapid stream of N_2. We agree, however, with Johnson [1933, 1] that the presence of a small persistent residue does not affect the end-point. Bessey [1938], on the other hand, considers it essential to effect complete removal of H_2S; and in all the tests to be described below a rapid stream of N_2 was passed for at least 6 hr.

Estimations in presence of SO_2 or Sn. SO_2, added as a preservative to fruit pulps and certain other food materials, may interfere, if present in large quantities, by reducing the dye. It may be removed by the two following methods:

To 20 ml. of the acid extract add 5 ml. acetone. This removes SO_2 as the sulphite derivative. Adjust the volume to 25 ml. The titration can then be carried out in the usual way, either visually or electrometrically [Harris *et al.* 1942]. As the acetone slows down somewhat the rate of the reaction between the ascorbic acid and the dye, carry out the titration more deliberately than normally, viz. let it take about 40–60 sec. (for further details of the acetone method see Mapson [1942]).

An alternative method is to pass a stream of O_2-free N_2 through the acid extract, while a vacuum is simultaneously applied (apparatus as in Fig. 1). Such treatment removes virtually all the free SO_2 within about 15–30 min. and the titration can then be carried out as usual. The above 'acetone' method is, however, to be preferred.

Usually the amount of SO_2 remaining after the preliminary grinding and extraction of the plant tissue does not exceed 50–100 parts per million. This will affect the result to the extent of about only 1–2 mg./100 g., which is of little consequence except with materials of low potency. There may be somewhat greater interference with fruit juices, since grinding is not employed and hence less SO_2 is lost.

Sn. The amount of tin normally present in fruits and vegetables, fresh, canned and household-cooked, is insufficient to interfere with the test. Only abnormally large quantities give trouble and these can easily be removed by preliminary treatment of the extract with H_2S, or by the method of Emmerie & van Eekelen [1934]. Then filter, or separate the supernatant liquid by spinning; remove the H_2S by simultaneous O_2-free N_2 plus vacuum, as above, and titrate in the usual way.

Example of calculation. 5 g. of cabbage taken, diluted to final volume of 100 ml. In titration 0·64 ml. of this extract matched 0·05 ml. of dye (0·1 ml. of dye $\equiv$ 0·04 mg. ascorbic acid). Hence ascorbic acid $= 0\cdot04 \times \dfrac{0\cdot05}{0\cdot1} \times \dfrac{100}{0\cdot64} \times \dfrac{100}{5} = 62\cdot5$ mg./100 g.

C. BIOLOGICAL ASSAYS

For the purposes of the present investigations it was considered advisable to concentrate our attention on those products which, supposing the objections set out at the beginning of the paper (p. 155) were valid, might be expected to give the least good agreement between the chemical and biological values. In this connexion three principal factors have to be considered. (1) The presence of 'combined ascorbic acid', not allowed for by the usual titration procedure, is postulated in certain vegetables (but not fruits), notably in potatoes. (2) The contribution of any dehydroascorbic acid to the total antiscorbutic activity has also to be considered, and potatoes again have been cited as among the

11-2

materials containing the most appreciable quantities of it, particularly after long storage. (3) 'Non-specific reducing substances' other than vitamin C may be produced when sugars are heated with alkali, and, it has been suggested, might occur therefore in certain processed articles, particularly those prepared with added sugar.

With these considerations in mind the following products were chosen for investigation: (1) freshly lifted potatoes, (2) potatoes stored for 5 months, (3) canned black currant purée consisting of sieved black currants cooked with sugar, (4) freshly picked black currants, (5) the 'cooking water' in which fresh cabbage had been boiled, and (6) a special dried cabbage prepared in such a way as to retain its activity towards the indophenol reagent. We were thus able to compare the results on a fresh vegetable with those on a stored vegetable (potato), and a fresh fruit with a preserved fruit (black currant). Black currants were included in the assay also for another reason; attention had first been attracted [Olliver, 1936] to their exceptional potency as a result of chemical tests by the method of Harris & Ray, and it was desired to have an additional check. By examining fresh black currants and stored potatoes, we were also able to compare a rich with a poor source of the vitamin. The 'cabbage water' was chosen because it might perhaps be argued that the large amounts of the reducing substance found in the cooking water were unlikely to be genuine vitamin C, and the dried cabbage because it provided a good example of a processed food whose biological activity might likewise be questioned.

Four series of biological assays were carried out.

Series 1 and 2, dental method

The histological 'tooth-structure' method was used for the biological tests in series 1 and 2. This method has already been found to give reliable results and to agree with tests by other biological procedures [e.g. Eddy, 1929; Key & Elphick, 1931; Harris et al. 1932; Harris & Ray, 1932; 1933, 2, 3, 4; Key & Morgan, 1933; Pierson, 1933; Coward & Kassner, 1936; Coward, 1938].

For both series 1 and 2, 50 young male guinea-pigs were placed for a few days on the basal scurvy-producing diet* supplemented with 15 g. of cabbage daily, until they weighed between 250 and 300 g. They were then divided into ten groups. One group was kept as negative controls and for the remainder the diet was supplemented with graded doses of the material under test or of the standard (ascorbic acid). Doses of 2·5, 1·25 and 0·6 mg. of ascorbic acid were administered daily to three groups, and the calculated quantities of potatoes and black currants equivalent to these levels of ascorbic acid were given daily to the remaining groups. These calculated amounts were based on the results of chemical determinations carried out daily on the materials under test, and thus the amount given varied slightly from day to day.

In the first series (July 1938) new Eclipse potatoes were tested and these were lifted from the plants on each day of dosing to ensure freshness of material. Ripe Baldwin black currants which were also tested in this series were picked daily from the bushes a few hours before each dosing. Owing to the very high concentration of ascorbic acid in black currants it would have been necessary to dose only a portion of a berry per day in the low-level group. Since also black currants are known to vary greatly from berry to berry in their concentration of ascorbic acid (Table 2), it was decided to reduce error on this score by mincing finely a large quantity of the fruit, straining, and diluting to 1 in 3 with water just before dosing. Duplicate chemical tests showed that in this way a homogeneous mixture was obtained.

To mitigate as far as possible any error due to variations in the composition of different potatoes and portions of potatoes, each guinea-pig was fed on small pieces of tissue cut from different parts of various potatoes. Each piece was cut immediately before dosing, in order to avoid oxidation.

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* The basal diet used both for this work and for the later tests by the growth method (series 3 and 4) had a composition similar to that described by Harris et al. [1932]:

Ground whole oats ('Sussex oats')	720 parts by weight
Bran	160　　,,　　,,
Dried egg yolk	90　　,,　　,,
Salt mixture	20　　,,　　,,
'Radiostoleum' (for vitamins A and D)	3 drops twice a week

In the most recent experiments of series 4, the whole ground oats became unobtainable and had to be replaced by ordinary oatmeal (kilned). The precaution was taken therefore of adding 10% dried brewer's yeast to the diet to make up for destruction of vitamin B_1 and possibly other factors during the kilning process.

Table 6. *Biological assays by tooth-structure method. Series 1, July 1938*

Material	Dose given, in terms of ascorbic acid, as determined chemically, mg.	Serial no. of animal	Score awarded			
			Lab. 1	Lab. 2	Lab. 3	Average
Potatoes, new, Eclipse	2·5	22	4	3·5	4	3·8
		50	?	4	4	4·0
		24	4	4	3·5	3·8
		2	4	4	4	4·0
		48	4	4	4	4·0
						Av. 3·9
	1·25	4	1·5	2	2	1·8
		33	3	3	3	3·0
		13	4	4	3·5	3·8
		17	4	4	3	3·7
		40	3	3	3·5	3·2
						Av. 3·1
	0·6	7	3	3·5	3·5	3·3
		43	1	1	1	1·0
		41	0	1	1	0·7
		23	1	1·5	1·5	1·3
		37	0	1·5	1·5	1·0
						Av. 1·5
Black currants, new, Baldwin	2·5	45	3	4	4	3·7
		47	4	4	3·5	3·8
		5	4	4	4	4·0
		8	4	4	4	4·0
		31	2·5	3	3	2·8
						Av. 3·7
	1·25	9	2·5	2	1	1·8
		16	4	4	4	4·0
		15	1	1	0	0·7
		19	4	4	4	4·0
		29	3·5	2·5	3	3·0
						Av. 2·7
	0·6	10	0	1	0·5	0·5
		36	1	1	1	1·0
		39	2	2	2	2·0
		34	1	1·5	1·5	1·3
		27	0	0	0	0
						Av. 1·0
Ascorbic acid	2·5	28	4	4	4	4·0
		38	4	4	4	4·0
		1	4	3	3	3·3
		14	4	4	4	4·0
		20	4	4	3·5	3·8
						Av. 3·8
	1·25	46	4	4	4	4·0
		49	?	0	0	0
		25	3	3	3	3·0
		21	4	4	4	4·0
		12	3	3	3	3·0
						Av. 2·8
	0·6	35	0	0	0	0
		30	3	3	3	3·0
		32	3	2	1	2·0
		42	2	1	1·5	1·5
		11	?	2	2	2·0
						Av. 1·7
Negative control	0	18	1	1	1	1·0
		26	1	1·5	1	1·2
		3	0	0	0	0
		44	0	0·5	0·5	0·3
		6	1	0	0	0·3
						Av. 0·6

Table 7. *Biological assays by tooth-structure method. Series 2, January 1939*

Material	Dose given, in terms of ascorbic acid, as determined chemically, mg.	Serial no. of animal	Score awarded			
			Lab. 1	Lab. 2	Lab. 3	Average
Potatoes, old, from clamp, Eclipse	2·5	43	4	4	4	4·0
		1	4	4	4	4·0
		6	4	3	3	3·3
		21	4	4	4	4·0
						Av. 3·8
	1·25	2	1	2·5	2·5	2·0
		3	0·5	0·5	0·5	0·5
		9	3·5	3	2·5	3·0
		37	3·5	3	3	3·2
		45	3·5	3	3	3·2
						Av. 2·4
	0·6	17	0·5	0	0·5	0·3
		23	2·5	2·5	2·5	2·5
		38	1	1	1	1·0
		44	0·5	0·5	0·5	0·5
		54	0	0	0	0
						Av. 0·9
Black currant purée (6 months after canning)	2·5	10	4	4	4	4·0
		22	4	4	4	4·0
		26	4	4	4	4·0
		27	4	4	4	4·0
		48	4	4	4	4·0
						Av. 4·0
	1·25	5	3·5	3·5	3·5	3·5
		13	3·5	3·5	3·5	3·5
		40	3	3	2·5	2·8
		46	2	2	1·5	1·8
		50	3·5	3	3	3·2
						Av. 3·0
	0·6	33	0·5	0·5	0·5	0·5
		34	3	3	3	3·0
		49	1	2	2	1·7
		51	0·5	1	1·5	1·0
		52	2	2	2	2·0
						Av. 1·6
Ascorbic acid	2·5	4	4	4	4	4·0
		24	4	4	4	4·0
		25	4	4	3·5	3·8
		28	4	4	3·5	3·8
		36	4	4	3·5	3·8
						Av. 3·9
	1·25	11	2	2	1·5	1·8
		16	3·5	3·5	3·5	3·5
		35	4	3·5	3·5	3·7
		41	2·5	2·5	—	2·5
		53	2	2	2·5	2·2
						Av. 2·7
	0·6	8	0·5	1	1	0·8
		12	1	1	1	1·0
		15	2·5	2	2	2·2
		47	1	1	1·5	1·2
		55	1	2	2·5	1·8
						Av. 1·4
Negative controls: (1)	0	14	0·5	1	0	0·5
		18	0	0	0	0
		19	0·5	0	0	0·2
		32	0·5	0·5	0·5	0·5
		42	?	0	0	0
						Av. 0·2
(2)	0	7	0	0	0	0
		20	1	0·5	0	0·5
		30	0·5	0·5	0	0·3
		31	0·5	0·5	0·5	0·5
		39	0	0	0	0
						Av. 0·3

At the end of two weeks' daily dosing, the animals were killed, the lower jaw removed and sections made of the decalcified incisors. Microscopic examinations of the sections for histological scorbutic changes were then made [Key & Elphick, 1931].

The second series (January 1939) of assays was conducted on similar lines, ascorbic acid, Eclipse potatoes and canned black currant purée being administered daily to the guinea-pigs. The potatoes were identical with those used in the first series but had been stored in a clamp for 5 months after lifting. The black currant purée had been made the previous summer from sieved black currants mixed with sugar and water and then canned and processed. This canned product had been stored for 6 months.·

Table 8. *Calculation of results of biological tests, series 1 and 2*

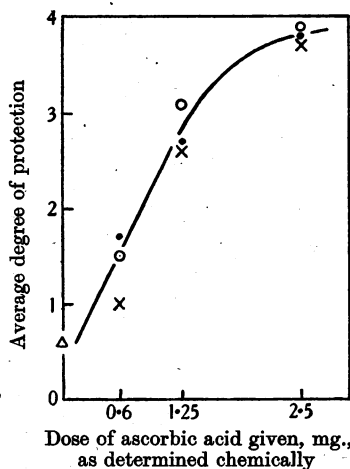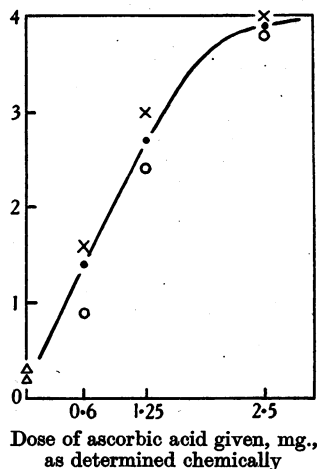| Description	Dose given, as determined chemically mg.	Biological activity (as determined from dose-response curve, Figs. 2, 3), mg.	Ratio of values biological : chemical
Series 1: Potatoes (new)	2·5	—	—
	1·25	1·4	1·12
	0·6	0·6	1·0
			Av. 1·06
Black currants (new)	2·5	—	—
	1·25	1·2	0·96
	0·6	0·4	0·67
			Av. 0·82
Series 2: Potatoes (old)	2·5	—	—
	1·25	1·1	0·88
	0·6	0·4	0·67
			Av. 0·78
Black currants (purée)	2·5	—	—
	1·25	1·4	1·12
	0·6	0·7	1·17
			Av. 1·15
Final averages: Potatoes			1·06
			0·78
			Av. 0·92
Black currants			0·82
			1·15
			Av. 0·99

Fig. 2.   Fig. 3.

Fig. 2. Dose-response curve, series 1. ○ = potatoes, new.   × = black currants, new.   ● = ascorbic acid standard.   △ = negative control.

Fig. 3. Dose-response curve, series 2. ○ = potatoes, old.   × = black currant purée.   ● = ascorbic acid standard.   △ = negative control.

Results. The findings in the first two series of tests are given in Tables 6 and 7. To reduce subjective errors, independent observers from three different institutes were asked to assess the degree of protection shown by the microscopic sections of the guinea-pigs' teeth, and the averages of the closely concordant findings were accepted.* The dose-response curves are presented in Figs. 2 and 3, and the final calculations of activities in Tables 8 and 12. It will be seen that, within the limit of biological error, there is good agreement between the results from the biological and the chemical methods in all instances. This agreement is all the more striking when it is realised that the daily doses administered to the animals varied from 0·6 g. of the highly potent black currant purée to 30 g. of the relatively inert stored potato.

Statistical accuracy of the biological tests. Miss H. Bruce has kindly submitted the results of the biological assays to a statistical analysis. She reports as follows. 'The *apparent-dose ratio* (i.e. ratio between the doses of a given substance as determined biologically and chemically) was calculated by the method of common slope [Irwin, 1937]. Since the highest dose in every instance gave a nearly maximal response, the results have been calculated from the two lower doses only, and the straight line relation is assumed to be between the log dose and the effect (Table 9). The significance of the differences between the results as determined by biological and chemical methods has been tested for each pair of doses (comparison being made with ascorbic acid at the same dose level in each case) by the application of Fisher's "t" test (Table 10). It is evident that none of the differences are significant and it may be concluded that there is complete agreement between the two methods of assay. In view of the excellent agreement between the three independent observers (cf. Fig. 4), it is clear that the subjective error in matching against the scale does not play so large a part as might have been supposed.'

Series 3 and 4, curative growth method

Method. The tests on 'cabbage water' and dried cabbage were done by the curative growth method [Harris *et al.* 1932; Harris & Ray, 1932; 1933, 1, 2, 3]. Only preliminary accounts of this method have hitherto been published, but Glazunov [1937], who compared it with the various alternative biological methods available, considered it to be the most convenient and accurate. The essential part of the procedure is to place standardized guinea-pigs, all males, weighing exactly 300 g., for 10 days on a scurvy-producing diet, and then on the 11th day of the experiment to begin the dosing of the various graded supplements. The 11th day is chosen as being that on which the sudden and precipitous drop in weight is about to begin, under the closely specified conditions. If larger animals are used, or higher reserves have been built up, dosing must be delayed by one or more days and *vice versa* depending on conditions, e.g. possible seasonal variations. The supplements, in other words, are given just in time to prevent any excessive loss in weight; the animals are already depleted (microscopic lesions in the dental structure have by then appeared), but there are still no obvious signs of deficiency. It is essential to time the beginning of the dosing to the nearest day, otherwise an animal which has already begun to lose weight rapidly will probably fail to respond satisfactorily or will only do so after undue delay. It is a useful precaution therefore to take the first few guinea-pigs in each test as 'pilot' animals, or negative controls, to indicate when the fall in weight sets in; this need not vary by more than 2–3 days under a given set of experimental, climatic or seasonal conditions.

To equalize the 'pre-experimental' conditions, the guinea-pigs are taken off their normal mixed diet when between 200 and 250 g. in weight and allowed 15 g. of cabbage daily in addition to the scurvy-producing diet, so as to build up their reserves to a uniform extent. This cabbage is withdrawn and the animals kept on the basal diet alone, starting from the exact day when each animal first reaches 300 g. in weight. It is found necessary during this preliminary period to weigh them daily, and determine the day on which the depletion period is to start separately for each animal, rather than for the group of animals as a whole.

* We are indebted to Miss H. Bruce of the Pharmaceutical Society's Laboratory for her participation in the team of assessors.

Table 9. *Statistical analysis. Calculation of ratio of biological to chemical results by method of common slope*

Series no.	Substance	Apparent dose ratio as calculated from biological test			
		Lab. 1	Lab. 2	Lab. 3	Mean of 3 observers
1	Potato, new	0·81	1·15	1·12	1·03
	Black currant, new	0·77	0·85	0·78	0·80
2	Potato, old	0·85	0·80	0·72	0·79
	Black currant purée	1·12	1·18	1·09	1·13

Table 10. *Calculation of 'significance'*

Series no.	Substance	Dose as determined chemically, mg. ascorbic acid	Value of 't'		
			Lab. 1	Lab. 2	Lab. 3
1	Potato, new	1·25	0·7	0·5	0·3
		0·6	0·9	0·1	0·3
	Black currant, new	1·25	0·8	0·1	0·4
		0·6	1·3	0·8	0·8
2	Potato, old	1·25	0·5	0·5	0·6
		0·6	0·5	1·2	1·4
	Black currant purée	1·25	0·5	0·7	0·08
		0·6	0·3	0·6	0·4
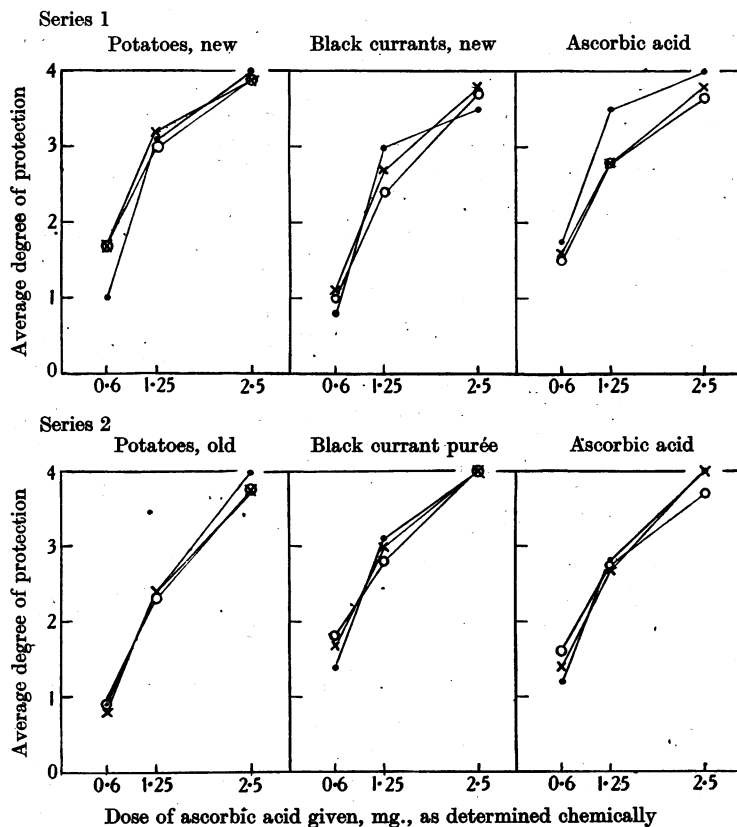
Fig. 4. Tooth-structure method: agreement between different observers. Dose-response curves, based on the assessments reached independently in three laboratories. Above, series 1; below, series 2.
●———●———● lab. no. 1.   ×——× × lab. no. 2. O———O———O lab. no. 3.

The basal diet is the same as that previously described (p. 164). With about 5–6 animals on each level of standard and unknown, the results have a very satisfactory degree of statistical significance [Harris, 1941].

Plan of experiments. In a preliminary trial run (Figs. 5, 6) graded doses of standard alone, 0, 0·2, 0·4, 0·6, 0·8 and 1·0 mg. ascorbic acid, were tested. This showed that suitable levels of the 'unknown' for test were those equivalent to 0·3, 0·5 and 0·7 mg. of ascorbic acid. For maximal accuracy the test could be continued for about 20 to 30 days after the 10-day depletion, although after only 10–15 days the dose-response curve (Fig. 6) is already sufficiently steep to permit a fairly close estimate. A further extension beyond 30 days is inadvisable, however, because of the loss in weight which then sets in, presumably owing to deficiency of some further unspecific factor.*

The assessment can be based on the loss or gain in weight during either the total period of the experiment, i.e. depletion period plus dosing period, or the depletion period alone. A possible advantage of the former alternative is that if the average growth curve of a group should happen to be below normal during the short depletion period, it is likely to be compensated for in the subsequent part of the curve; i.e. by taking the longer period for comparison, initial random variations are more likely to even themselves out. Examples of both methods of reckoning are shown in the figures, and it is clear that the values obtained by either method are virtually identical provided they are based on a sufficient number of experimental data; in individual assays the method giving the smoother dose-response curve may be legitimately selected.

In series 3 (June 1941) the three levels of ascorbic acid referred to above, 0·3, 0·5 and 0·7 mg. were given daily together with the calculated equivalents of 'cabbage water', as determined chemically by titration, there being 6 animals in each of the 6 groups. (To prepare the 'cabbage water' 100 g. of fresh cabbage were placed in 250 ml. of boiling freshly distilled water and cooked for 20 min.; the water was cooled and poured off, titrated for ascorbic acid and made up to a dilution such that portions of 1·2, 2·0 and 2·8 ml. respectively gave the three doses required.) In series 4 (August 1941) the same biological procedure was used for testing the dried cabbage, the same calculated equivalents, 0·3, 0·5 and 0·7 mg., being administered daily, and the same doses of standard.† The average weight curves and the corresponding dose-response curves for series 3 are given in Figs. 7 and 8,

Fig. 5. Curative growth method. Average weight curves of groups of guinea-pigs receiving 0, 0·2, 0·4, 0·6, 0·8 and 1·0 mg. of ascorbic acid daily. Six animals in each group. No ascorbic acid until eleventh day of experiment when dosing started (marked by dotted line).

respectively, and for series 4 the dose-response curve only is shown in Fig. 9.

Results. A glance at the dose-response curves (Figs. 8, 9) shows clearly enough the good agreement between the calculated and the observed responses. The computation
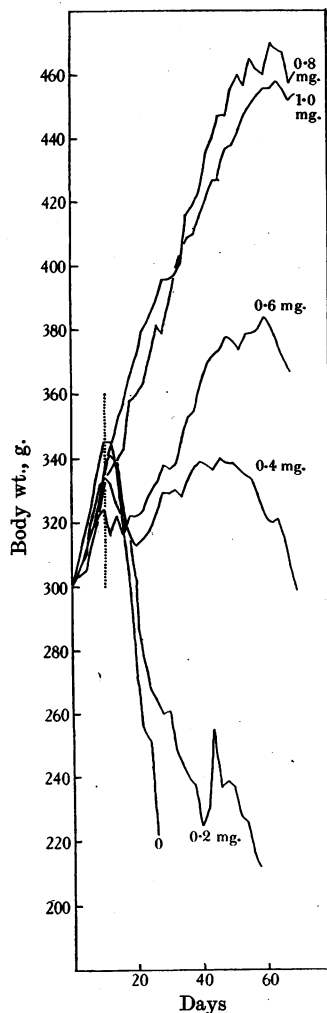
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* Attempts to restore weight by addition of extra yeast, vitamin E etc. were unsuccessful.

† The test on the dried cabbage was done in collaboration with Dr L. W. Mapson in the course of investigations on processed foods, carried out on behalf of the Food Investigation Board. We are indebted to them and him for permission to refer to it here.

of the exact biological values is given in Table 11, and it will be seen that these differ from the chemical values by less than 1 %.
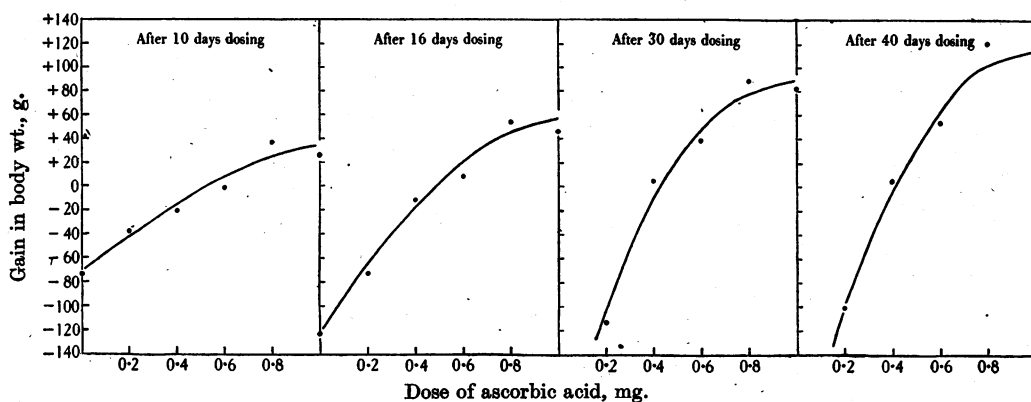

Fig. 6. Dose-response curves corresponding with Fig. 5, after 10, 16, 30 and 40 days of dosing. Losses or gains in weight are here reckoned from beginning of dosing. (Similar curves are obtained if losses or gains are reckoned from beginning of depletion: cf. e.g. Fig. 8.)
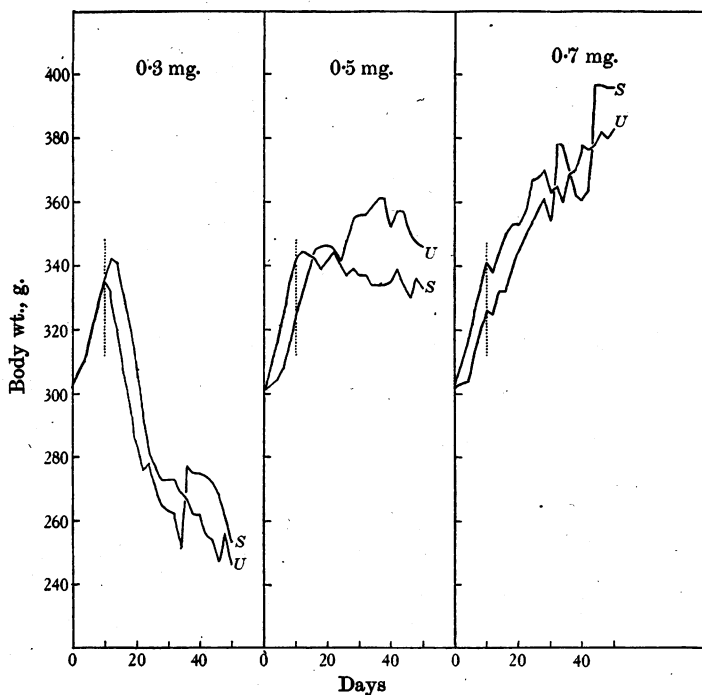

Fig. 7. Average weight curves of guinea-pigs, receiving 0·3, 0·5 and 0·7 mg. of ascorbic acid and the corresponding equivalents of 'cabbage water' as determined chemically. Six animals per group. Dosing begins after 10 days' depletion (dotted line). S = Standard (ascorbic acid). U = 'Unknown' (cabbage water).

SUMMARY OF BIOLOGICAL TESTS

In Table 12 are assembled final comparisons of the chemical and biological determinations. The products examined were very varied in character and covered a wide range of potency (from 9 to 460 mg. of ascorbic acid per 100 g.). The average of the biological
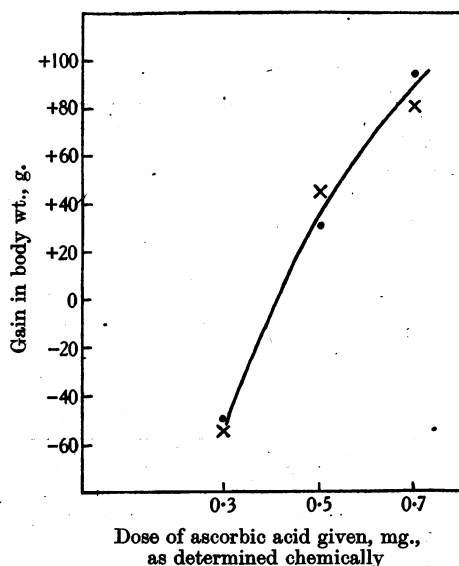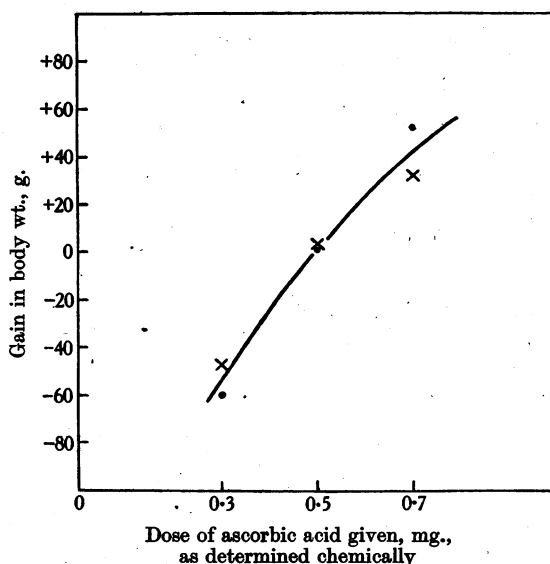
Fig. 8.   Fig. 9.

Fig. 8. Dose-response curve for 'cabbage water', corresponding with the growth curves in Fig. 7. (Values after 40 days' dosing. Depletion period included in the reckoning of gains or losses; similar result obtained if excluded, cf. Fig. 6.) ● ● ● =standard (ascorbic acid). × × × =unknown (cabbage water).

Fig. 9. Dose-response curve for dried cabbage. Six animals per group. Results after 20 days' dosing; values calculated from beginning of depletion period (cf. Fig. 6). ● ● ● =standard (ascorbic acid). × × × =unknown (dried cabbage).

Table 11. *Calculation of results of biological tests, series 3 and 4*

Description	Dose given, as determined chemically mg.	Biological activity, as determined from dose-response curves, Figs. 8, 9 mg.	Ratio of values, biological : chemical
Series 3, cabbage water	0·3	0·295	0·98
	0·5	0·53	1·06
	0·7	0·665	0·95
			Av. 1·00
Series 4, dried cabbage	0·3	0·32	1·07
	0·5	0·51	1·02
	0·7	0·65	0·93
			Av. 1·01

Table 12. *Summary of chemical and biological comparisons*

Description	Method used for biological assay	Average content of ascorbic acid, mg./100 g. Determined chemically	Determined biologically	Biological value, as % of chemical value
Black currant (new)	Tooth structure	237	194	82
Black currant (purée)	„ „	120	138	115
Potatoes (new)	„ „	35	37	106
Potatoes (old)	„ „	9	7	78*
'Cabbage water'	Curative growth	25	25	100
Dried cabbage	„ „	460	463	101
			Av.	97

* Low value attributed to incomplete absorption; very large doses needed in biological tests.

values is 97 % of that of the chemical values. This agreement seems very satisfactory, especially when it is borne in mind that what small difference there is, is almost certainly due to the liability for low biological values to be given by relatively inert products such as old potatoes, since these had to be administered in unduly large doses and thus the vitamin was likely to be incompletely absorbed. Incidentally, our figures seem to confirm the conclusion of Glazunov [1937] that, with proper precautions, the curative-growth method is considerably more sensitive and certainly less troublesome than the microscopic tooth-structure method.

D. SUPPOSED OBJECTIONS TO THE TITRATION METHOD

At the beginning of this paper three alleged sources of error in the titration method were mentioned; these may now be considered in turn.

(1) Interfering substances

It has been suggested that the results obtained by the chemical method may possibly be too high owing to the presence of substances other than ascorbic acid which may reduce 2:6-dichlorophenolindophenol. This criticism has been mostly levelled against the method as applied to animal tissues. Emmerie & van Eekelen [1934] proposed to insert a preliminary treatment with mercuric acetate to remove such reducing substances, and several workers have applied this modification when testing plant tissues. We are not aware, however, of any experiments which have demonstrated the presence of significant amounts of such interfering substances in plant tissues, and the proposed modification of Emmerie and Eekelen is considered inadvisable by most investigators [e.g. Bessey, 1939: see also below, Discussion p. 179].

Sugar derivatives. Products formed by the treatment of certain sugars with hot alkali, or other chemical agents, may under some circumstances reduce the dye [Harris, 1933]. It seems certain, however, that such substances are not produced by the methods ordinarily used for the 'processing' of fruits and vegetables, i.e. in cooking, canning or jam making. In control experiments, solutions of sugars and of acids, in concentrations similar to those normally added to (or present in) fruits and vegetables, were canned and processed alone, and it was observed that there was no development of reducing power towards 2:6-dichlorophenolindophenol. Again, fruits and vegetables initially low in ascorbic acid were found to have equally negligible values after canning, while the more active raw materials

Table 13. *Reliability of chemical method as illustrated by values obtained for jams: products found to be low in ascorbic acid are those made from fruits of known low potency, and vice versa*

	Ascorbic acid content, mg./100 g.			Ascorbic acid content, mg./100 g.	
Variety	In fruit used	In jam prepared	Variety	In fruit used	In jam prepared
Black currant	144	45	Raspberry	19	8
Strawberry	64	25	Greengage	3	<1
Red currant	30	13	Red plum	2	1
Marmalade	42	10	Apple jelly	2	<1
Gooseberry	40	11	Golden plum	2	<1

yielded correspondingly active products. Similarly, negligible values were found for jams and jellies made from fruits initially low in vitamin C, and *vice versa*. For example, plums, greengages and apples are fruits relatively low in vitamin C, and it was found that plum and greengage jams and apple jelly prepared under normal conditions of manufacture, had reduction values towards 2:6-dichlorophenolindophenol corresponding to less than 1 mg. of ascorbic acid per 100 g. Other examples of this parallelism are shown in Table 13.

Reference was made above (p. 163) to the need for removing SO$_2$ and Sn if present in large amounts as contaminants of food materials. We are not aware of any other inter-

fering substance likely to be found in ordinary fruit or vegetable preparations, or occurring naturally in the plant tissues. The nature of certain reducing substances in malt extracts, in yeast and fermented juices and in mushrooms will be referred to below.

(2) *Dehydroascorbic acid*

In their pioneer work on the reducing action of plant juices towards the indophenol dye, Tillmans et al. [1932, 1, 2] found that they often had to include a preliminary treatment with H_2S in order to regenerate the reducing principle (now recognized as ascorbic acid) from its reversibly oxidized form. It now seems certain that the explanation is that during their laborious, and on the whole unsatisfactory, process of preparing the extract extensive oxidation must have occurred. When a little later the method for the actual quantitative estimation of the vitamin was worked out at Cambridge, employing an improved method of extraction with trichloroacetic (or, afterwards, metaphosphoric) acid, the treatment with H_2S was found to be unnecessary, for the titration values in the absence of H_2S agreed excellently with the biological assays [e.g. Harris & Ray, 1933, 1; Birch et al. 1933; Harris, 1937].

McHenry & Graham [1935, 1], and Reedman & McHenry [1938], have made the surprising claim that considerable quantities of reversibly oxidized ascorbic acid are present in fresh vegetables. This is in conflict with the finding of nearly all recent observers, and we believe that here again the explanation is that oxidation occurred during the extraction. It is noteworthy that McHenry and his co-workers employ an elaborate process of extraction which would conduce to oxidation. Moreover, the values which they report for reduced ascorbic acid are so extraordinarily low compared with those found by other workers, whether using biological or chemical methods in which oxidation is better guarded against, that there seems little doubt that the large amount of reversibly oxidized ascorbic acid is indeed an artefact. Our experience is in line with that of Stone [1937], who was unable to detect any dehydroascorbic acid in the intact vegetable and believes it to be produced when the cellular tissue is broken and exposed to the air, and that of Bessey [1938], who found no significant quantity of dehydroascorbic acid in fresh vegetables and only small quantities in stored or wilted vegetables.*

Experimental. Our own extensive determinations, entailing many hundreds of titrations on numerous fruits and vegetables, lead us to confirm the original view of Harris & Ray. The small traces of dehydroascorbic acid found in fresh plant tissues sink into insignificance compared with the effect of other variables already mentioned in this paper, i.e. variations between one sample and the next, and between different portions of the same specimen, and between one batch and another. Compared with the great natural range of activities in the plant kingdom—extending, for example, from such high values as hips (500 mg./100 g.) or black currants (200), through medium figures such as cabbage (100) or lemon (50), down to the low level of pears (3) or plums (2)—it is obvious that the presence of a mere 1 or 2 % in the form of dehydroascorbic acid can be disregarded as of little or no practical consequence to the dietitian. A few representative figures from our analyses are given in Table 14. It will be seen that, among the materials we examined, only stored potatoes had any significant amount of dehydroascorbic acid. Even so, the agreement between the results obtained biologically and those by the chemical method without previous reduction (Fig. 2) would suggest that for all practical purposes the treatment with H_2S is scarcely necessary.

* Ascorbic acid oxidases are generally found in vegetable rather than in fruit tissues, and it is not surprising therefore that the majority of workers have found dehydroascorbic acid to be associated with vegetable extracts rather than with fruit juices. Zilva et al. [1938], on the other hand, found about 50 % of the total ascorbic acid of immature apples to be present in the reversibly oxidized form. The proportion diminished as the fruit approached maturity. This is an interesting point which would no doubt repay further investigation.

Table 14. *Dehydroascorbic acid in fruits and vegetables*

Material	No. of batches tested	Ascorbic acid, mg./100 g.		Dehydroascorbic acid			
				mg./100 g.		As % of total ascorbic acid	
		Range	Average	Range	Average	Maximum	Average
Asparagus	12	50–97	74	0–4	1	6	1
Black currants	8	188–289	233	0–14	3	5	1
Broccoli (white head)	12	57–79	71	0–3	<1	4	1
Cauliflower (white head)	9	49–92	74	0–3	<1	2	<1
Gooseberries	8	38–48	46	Nil	—	Nil	—
Peas	7	20–38	29	0–3	1	8	1
Potatoes: New	14	30–36	33	0–2	0·5	6	1
Stored*	23	8–17	11	0–1	<1	12	6

* All material except stored potatoes was freshly gathered before testing.

Table 15. *Apparent dehydroascorbic acid content of black Leicestershire mushrooms*

Time of addition of acid after commencement of grinding min.	Ascorbic acid, mg./100 g.		Time of addition of acid after commencement of grinding min.	Ascorbic acid, mg./100 g.	
	Determined without reduction by H_2S	Determined after reduction by H_2S		Determined without reduction by H_2S	Determined after reduction by H_2S
0	3	10	0	3	6
0	3	8	½	3	14
0	3	15	1	3	19
0	4	18	2	3	15

Anomalous results with mushrooms. Contrary to the general rule, we observed that with mushrooms a considerable rise in reducing value occurred after treatment with H_2S (Table 15). This increase cannot presumably be due to the action of oxidases during the grinding, since previous grinding before the addition of acid did not affect the results (cf. Table 3). The presence of preformed dehydroascorbic acid in mushrooms seems possible because of their large surface (porous nature of tissues) and the occurrence of oxidase systems. It was significant, however, that with the extracts from mushrooms the end-points in the titrations were exceptionally poor, and faded rapidly. It seems likely, therefore, especially when it is borne in mind that mushrooms are so different physiologically and morphologically from other fruits and vegetables, that this acid-extract contains additional substances distinct from dehydroascorbic acid, which after reduction with H_2S will decolorize the dye. Mack & Tressler [1937], working with other plant extracts, have suggested that an apparent increase in reducing substances after treatment with H_2S is not always due to dehydroascorbic acid. In view of this doubt, and bearing in mind the small practical significance of dehydroascorbic acid for fruits and vegetables in general, we are once again forced to the conclusion that for routine estimations the preliminary reduction with H_2S is not necessary. Even when it is tried, as for exploratory experiments, the results must be interpreted with caution.

(3) Combined ascorbic acid

It was claimed by Ahmad [1935] that when plant tissues were heated an apparent increase in the amount of ascorbic acid could be observed. To account for this supposed increase, McHenry & Graham [1935, 2] advanced the theory that raw vegetables contain some of their ascorbic acid in the 'bound' form and that this is unable to reduce the dye; when the vegetables are cooked 'free' ascorbic acid is released and can thus be titrated. This alleged increase on cooking is, however, contrary to the experience of most workers, who find that heating diminishes rather than augments the amount of ascorbic acid. Van Eekelen [1935; 1938], working with potatoes, was unable to observe any increase on heating. He, and Harris [1937] both attributed the apparent increase on heating to the destruction of the natural ascorbic oxidases of the plant; unless proper precautions are taken these oxidases will inactivate the vitamin during the preliminary process of grinding and extracting before the titration.

   Later, this theory of 'bound ascorbic acid' has formed the subject of controversy. Thus, Guha & Pal [1936] claimed to have isolated 'bound ascorbic acid' from cabbage; but their findings were controverted by Mack [1936]. Levy [1936] at one time believed he had demonstrated the presence of 'bound ascorbic acid' in cauliflower and potatoes;

according to Mack & Tressler [1937], on the other hand, an apparent increase observed when certain extracts are heated may be sometimes due to the formation of physiologically inactive degradation products. Guha & Sen-Gupta [1938] supported the earlier work of Guha & Pal [1936], and Scarborough & Stewart [1938] reported that they found combined ascorbic acid in cauliflower juice; but as against this Bessey [1938] was unable to detect it either in cauliflower or in potato, and Levy [1937], in a later paper, finds that cooking causes a loss in ascorbic acid and not an increase, while Fujita & Ebihara [1938; 1939] admit that the case for combined ascorbic acid is 'still not proved' or 'could not be substantiated'.

Experimental: effect of cooking on ascorbic acid content. Applying the method for estimating ascorbic acid described in the present paper, we have made a prolonged series of tests, comparing numerous raw fruits and vegetables with the heated products. As a result we have been unable to observe an increase in ascorbic acid in the cooked products. Some typical results are given in Table 16.

Table 16. *Effect of cooking on ascorbic acid in fruits and vegetables*

Material	Raw tissue	Ascorbic acid, mg. per sample. Cooked			% loss on cooking
		Tissue	Liquor	Total	
Black currants	775	326	400	726	6
Broad beans	127	35	84	119	6
Broccoli	341	156	168	324	5
Cabbage	208	47	95	142	32
Cauliflower	172	68	98	166	3
Gooseberries	81	38	40	78	4
Peas	246	95	132	227	8
Plums	17	8	9	17	0
Potatoes	310	190	70	260	16
Spinach	114	47	23	70	38
Sprouts	160	52	97	149	7
Stringless beans	40	17	20	37	7

Apparent increase of vitamin C during cooking of carrots. At one time we were puzzled by the observation that cooked carrots seemed to be richer in vitamin C than the raw carrots from which they were prepared. The explanation of the supposed anomaly is instructive, and we believe worth recording here since the conditions must have been similar to those in experiments by other observers in which an apparent increase has been claimed. The carrots had been sliced in the usual way immediately before being dropped into the boiling water for the cooking, a portion of the same sliced carrot being also used for the control test on the raw material. The essential point which was at first overlooked was that the destruction of vitamin C by the ascorbic oxidases set free on slicing was inhibited in the cooked carrot the moment the freshly cut slices were dropped into the boiling water, whereas in the unheated control the oxidation proceeded for a short time before the slices were placed under the acid for extraction. When the experiment was more suitably controlled, the slicing of the raw carrots being done under the surface of the protecting acid before the grinding was started, it was found that cooked carrots had a lower and not a higher value than raw carrots. It should not be forgotten, however, that from the point of view of the dietitian there may in fact often be more vitamin C in cooked vegetables than in raw if the latter have been shredded or chopped before consumption.

Probable explanation of the 'combined ascorbic' hypothesis. We believe that three causes have combined to produce the misleading appearance of the increase in ascorbic acid after heating, recorded by McHenry and others, and responsible for the 'combined ascorbic acid' theory.

(a) *Incomplete extraction.* From unheated tissues, particularly if they are of a hard or fibrous nature, quantitative extraction of ascorbic acid is more difficult than from softened, heated materials. Thus a fictitiously low value is given to the raw product unless a satisfactory method of extraction is used.

(b) *Variations in sampling.* We have already stressed the importance of representative sampling, in order to assess the natural range of variations for a given natural product. Failure to do so will materially affect the correctness of the final conclusions.

In much of the published work on the effect of cooking, a single determination only is made on the raw material and another single determination on the cooked preparation. A slightly higher value for the cooked product is sometimes taken to mean a rise in ascorbic acid, although actually the difference observed is well within the natural range of variations.

(c) *Action of oxidases.* The most important factors are probably the ineffective procedures used to prevent the action of oxidases during the preliminary extraction of the raw material and the destruction of these same oxidases by the cooking or heating process. There is direct internal evidence that this cause has been at work, for (1) the method of extraction used by McHenry and Graham is calculated to permit oxidation of the raw material, and (2) the values for the raw materials as given are extraordinarily low compared with those obtained by other workers using both biological and chemical methods.

Experimental evidence: effect of faulty methods of extraction. We have confirmed the last-mentioned deductions given under heading (c) above by means of direct controlled experiments. Using the method of McHenry and Graham, which entails the use of large amounts of the material and a lengthy and laborious process of extraction, the values we obtained for ascorbic acid in various vegetables were considerably lower than with our own rapid process which safeguards effectively against such losses (Table 17). That oxidation occurs during McHenry's process is clear from the fact that a portion of the ascorbic

Table 17. *Effect of method of extraction on the total ascorbic acid content of vegetables. Ascorbic acid, mg./100 g.*

Material	Method of Harris and co-workers		Method of McHenry and Graham, using large quantities of tissue and laborious extraction	
	Before treatment with H_2S	After treatment with H_2S	Before treatment with H_2S	After treatment with H_2S
Asparagus	62	62	58	58
	66	66	56	56
Broccoli	64	64	35	39
	77	77	66	66
	73	73	55	59
Potatoes (old)	8	9	6	7
	7	7	2	5

acid can generally be recovered as reversibly oxidized dehydroascorbic acid, whereas with our method virtually none is to be found. Obviously heating of the vegetable, by inactivating the oxidase, will reduce oxidation and so lead to an apparently higher value for the ascorbic acid.

Reedman and McHenry's 'hydrolysis' method. Reedman & McHenry [1938] have stated that there are appreciable amounts of 'combined ascorbic acid' in potatoes, and have suggested a method of estimation based on a preliminary hydrolysis with HCl of the postulated protein-ascorbic-acid complex. We attempted to repeat their work, but at the start experienced difficulty in preventing oxidation during the hydrolysis with HCl in an atmosphere of CO_2, even at 40°, and found an atmosphere of H_2S to be more efficient. McHenry, in a private communication, confirmed this finding and also mentioned that a concentration of 0·2% HCl was preferable to one of 1%. We suggested that in carrying out the estimations of 'total ascorbic acid', a saving might be effected by combining all the extraction processes, i.e. first carrying out the hydrolysis with HCl, extracting the residue with trichloroacetic acid and metaphosphoric acid, combining the extracts and making a single titration. McHenry agreed with this and consequently the following procedure was adopted. The material was ground with 50 ml. of 0·2% HCl and transferred to a small wide-mouthed flask. H_2S was passed into the solution for 10 min. and the flask was stoppered and kept at 40° for 1 hr. The solution was then squeezed through muslin into a 250 ml. wide-mouthed flask graduated at 100 ml. The residue was extracted with 12·5 ml. of 20% trichloroacetic acid and 5 ml. of 20% metaphosphoric acid and washed into the flask, the volume being kept below 100 ml. H_2S was passed through for a further 5 min., the flask stoppered and left overnight. The H_2S was removed in a stream of N_2 and the volume made up to 100 ml. in a

graduated flask. The solution was then filtered and titrated. This titration gives the total ascorbic acid in the material; i.e. the reduced, reversibly oxidized and 'combined' forms of the acid. Using this method we have carried out extensive tests on potatoes, black currants, broccoli and asparagus and in no case has the hydrolysis method described given higher values than those obtained by the ordinary titration method, except in isolated instances which could be accounted for by the wide variation in the ascorbic acid content of the material under examination. Indeed, the hydrolysis method has in most instances given lower values than the direct method advocated in this paper. Representative figures are given in Table 18. It is probable that oxidation of free ascorbic acid occurs during the preliminary hydrolysis with HCl, since, provided that metaphosphoric acid is added to the hydrolysing acid, the final results agree well with those obtained by the usual direct method (Table 19).

Table 18. Comparison of 'direct' and 'hydrolysis' methods.
Ascorbic acid, mg./100 g.

Material	'Direct' method of Harris and co-workers (technique as in this paper)		'Hydrolysis' method, after Reedman and McHenry (atmosphere of H_2S)
	Before treatment with H_2S	After treatment with H_2S	
Asparagus	50	52	36
	53	56	40
	70	70	61
	75	76	61
	97	101	82
	97	97	92
Black currants	207	207	190
	146	148	149
	140	142	147
Black currants, minced and diluted for dosing	66	68	65
	66	66	64
	53	53	51
Peas	23	24	22
	23	·23	23
Potatoes (new)	29	29	27
	33	33	26
	30	30	25
	33	33	31
	27	28	27
	38	38	36
Potatoes (stored)	11	12	10
	10	10	10
	8	9	9

Table 19. Effect of addition of metaphosphoric acid to the hydrolysing acid in the method after Reedman and McHenry. Ascorbic acid, mg./100 g.

Material	Direct method		'Hydrolysis' method after Reedman and McHenry (atmosphere of H_2S)	As in last column but metaphosphoric acid added to hydrolysing acid
	Before treatment with H_2S	After treatment with H_2S		
Asparagus	72	74	61	71
	90	90	82	90
	97	97	92	96
	53	56	40	56
Broccoli	79	79	62	77
	77	77	62	72
	75	78	63	74
Potatoes (stored)	14	14	9	11
	14	14	12	13

DISCUSSION

Comparison of chemical and biological findings. The excellent agreement between the titrations and the biological assays, either as previously recorded or as newly determined here, is we believe the best evidence of the reliability of the chemical method. When it is

remembered that the activities of these common fruits and vegetables cover an extra-ordinarily wide range, from so high a potency as hip berries ($=500$ mg./100 g.) down to so low a figure as plums or pears (2–3 mg./100 g,), and that the chemical and biological results run side by side over this wide range, the correspondence becomes even more impressive. Results by the electrometric method [Harris *et al.* 1942] also agree with those by the colorimetric method to within 1 or 2 %; more significant, however, is the fact that further investigations at the Nutritional Laboratory by the method of polarographic analysis, depending on a different principle from indophenol titrations, again show a complete agreement with the latter [Kodicek & Mapson, 1942]. In practice the chemical method has the distinct advantage over the biological method, that, apart from being so much more rapid and convenient, it permits relatively small variations in activity to be detected. It can also be applied to far smaller specimens, which is a decisive factor in studies in plant physiology.

'*Combined ascorbic acid*'; *dehydroascorbic acid.* We have seen that it is important that the correct procedure be followed for the chemical test; otherwise errors may arise from at least three causes, viz. (1) unrepresentative sampling, (2) incomplete extraction, and (3) oxidative destruction of the vitamin. We believe that a failure to take such precautions is responsible for the conclusions of that small minority of workers who have contended that the 'reversibly-oxidized', or the supposed 'combined', forms of vitamin C constitute an important fraction of the total antiscorbutic activity of common foodstuffs. In particular, oxidation by ascorbic oxidases set free during the course of extraction (or by Cu, or other agencies) seems to have been responsible for the misleading appearance of 'combined ascorbic acid'. The simple explanation is, we believe, that low values were obtained by these observers on their raw materials because oxidation was not effectively prevented, and higher values after cooking because the oxidative enzymes had been thereby de-stroyed. As we have shown, there is internal evidence in the published records of such destruction by enzymes having actually occurred, e.g. in the abnormally low figures ad-vanced for the 'free ascorbic acid' of the raw materials. In our hands, attempts to repeat the published observations on 'combined ascorbic acid' have been uniformly unsuccessful whenever we took adequate precautions to prevent oxidation. At the same time we wish to emphasize that we have no intention of disputing the importance or the interest of the several papers by McHenry, and others; our interpretation of his results, however, is dif-ferent from his own since we believe that the apparent 'increase on cooking' is in reality an artefact.

Thus our general findings accord fully with those of van Eekelen [1935], who concluded that 'the apparent increase observed by McHenry and Graham must be attributed to the destruction of an oxidase present in some vegetables and not to the liberation of more ascorbic acid', or of Bessey [1939] who writes:

'Reversibly oxidized ascorbic acid (dehydroascorbic acid) represents an insignificant portion of the total biological value of fresh or stored food products.

'The titration value of some tissue extracts may be increased by preliminary treatment with hydrogen sulphide. Some investigators have interpreted this increase as due to reduction of dehydroascorbic acid. There is some doubt as to how much of this increase is due to the reduction of other substances which are then included in the titration. The uncertainty of the procedure is also increased by the necessity of completely removing the gas because hydrogen sulphide also reduces the indicator. Emmerie and van Eekelen recommended the use of mercuric acetate to remove cysteine and ergothioneine, and of thiosulphate followed by treatment with hydrogen sulphide. The extra manipulation involved, with uncertainty at every step, makes the method at present of doubtful value.'

We agree also with Levy [1937], who finds that cooking of vegetables causes a diminution, not an increase, in their ascorbic acid, and with Fujita & Ebihara [1938; 1939], who take the view that the theory of 'bound ascorbic acid' lacks proof. If any considerable portion of the ascorbic acid in fruits and vegetables is in fact present in some ill-defined 'bound' or 'combined' (e.g. adsorbed) state, it is apparent that the combination must be broken

up by the process of extraction used by ourselves and the titration gives an accurate indication of the total antiscorbutic activity.

Interfering substances.   Theoretically, any of the following substances can reduce the indophenol reagent under appropriate circumstances: ferrous salts, glutathione, cysteine, ergothioneine, sulphides, thiosulphates, tannin, glucoreductone, and possibly various sugar derivatives. However, it is well known that under the conditions of our test (i.e. preliminary extraction, acid reaction, rapid titration) glutathione and iron salts do not interfere. It is also equally obvious from our results that the other substances mentioned in this list are either not present in our extracts in the free form, or not in significant amounts, or else do not interfere, at any rate for any common fruits or vegetables, or fruit or vegetable preparations (including preserved, or dried or processed products).*

Special products.   Although the amount of dehydroascorbic acid present in fruits and vegetables is too small to be of any practical significance in nutrition, further investigation may perhaps be merited in the special cases of immature apples [Zilva *et al.* 1938] and mushrooms (*vide supra*, pp. 175 and 176). In addition, the reducing substances present in small amounts in germinated seedlings [Johnson, 1933, 2; but cf. van Eekelen *et al.* 1934], in malt extracts, and in yeast and fermented juices [cf. Harris, 1933] might repay study.

'Apparent biological value' and true ascorbic acid content.   A final point which should be emphasized is that in the cases of fibrous or woody vegetable tissues, or indigestible animal tissues, the vitamin may be incompletely absorbed by the test guinea-pig, and hence the 'apparent biological value' is less than the ascorbic acid content as determined chemically. An example is tumour tissue. Biological tests were consistently lower than the chemical values [Harris, 1933], and later work proved that this was largely, if not entirely, due to the inability of the guinea-pig to assimilate completely the untreated tissue [Kellie & Zilva, 1936; Musulin *et al.* 1936; Watson, 1936; Young, 1936; Harris, 1937]. It is important therefore for biological testing when dealing with such products to consider whether the vitamin should first be extracted or the food made digestible before it is administered to the animal.

SUMMARY

1. *Biological comparisons.*   The agreement between the results obtained by the chemical titration method and in biological assays has again been confirmed, in tests carried out on fresh potatoes, stored potatoes, freshly gathered black currants, canned sieved black currants cooked with sugar, cabbage 'cooking water' and dried cabbage (materials specially chosen to illustrate supposed objections to the method), and on other fruits and vegetables.

2. *Chemical procedure.*   Since it is necessary that conditions be adhered to strictly, detailed working directions are given for the chemical method, emphasis being laid on the necessity for the following precautions: representative sampling; complete extraction (facilitated by the use of a small specimen); inactivation of oxidases and prevention of oxidation; the titration itself to be rapidly carried out.

* In the case of animal products (to be discussed in detail in a later paper) interference from the fore-mentioned substances seems equally unlikely, certainly so far as concerns the general run of samples likely to be encountered in ordinary analytical practice or in biochemical research. The amino-acids mentioned are not likely to be present in the free state, except conceivably in autolysed preparations, and fortunately even these can be distinguished if necessary by the fact that the reaction between ascorbic acid and the dye is so much more rapid, in fact virtually instantaneous; a procedure can be applied depending on the addition of an excess of dye and instantaneous measurement of the excess of colour before interference from any non-specific reductants begins. Alternatively, in the unlikely event of free cysteine being present, it could if desired be measured differentially by the Sullivan reaction, or could be removed together with other interfering amino-acids before titration. Thiosulphate, present in urine in small amounts, does not interfere with the validity of saturation tests for determining the level of nutrition, these depending on an immense increase of true ascorbic acid after loading [see Harris, 1940]. In practice biological tests on animal tissues have shown close agreement with chemical tests.

3. '*Bound ascorbic acid.*'   The alleged increase of ascorbic acid after cooking, supposed to represent a release of 'bound ascorbic acid', could not be confirmed. Faulty technique is held responsible for such an apparent rise: e.g. (1) unduly low values have been recorded for the raw materials because of failure to protect against the action of oxidases set free on grinding and shredding, and higher values are found after cooking because the enzymes are thereby inactivated; or (2) the vitamin is sometimes less readily extracted from raw fibrous foods than from the cooked products. The method advocated measures the total antiscorbutic activity; if ascorbic acid exists in any combination it is broken down by the extraction process used.

4. *Interfering substances.*   Interfering substances were not found in measurable quantities in any fresh fruits or vegetables, or various processed materials. Large amounts of SO_2 used as preservative, or abnormal contamination with tin, can both be accurately differentiated from ascorbic acid, if present.

5. *Dehydroascorbic acid.*   The quantity of dehydroascorbic acid normally found in fruits and vegetables was so small as to be of little or no practical significance. The relatively large amount reported by a small minority of workers is attributed to failure to guard against oxidation during the preparation of an extract.

6. *General conclusion.*   Provided that the specified precautions are taken direct titration of the acid extract against 2:6-dichlorophenolindophenol can be recommended with confidence for all ordinary routine analyses of plant materials, as giving the total antiscorbutic activity. Tables are given showing the close correspondence between the chemical and the biological values.

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